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Time Resolved X-ray Diffraction Studies of Active Bony Fish Muscle: Analysis using the New CCP13 Program FibreFix

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The mechanism by which muscles generate force is thought to occur through the cyclical interaction of myosin crossbridges with the protein actin. Actin and myosin form separate filaments within muscles: the thin filaments contain actin and the thick filaments contain myosin. In the contractile cycle, the crossbridges or myosin heads, which protrude at regular intervals along the thick filaments, attach to the thin filaments and undergo a conformational change associated with the hydrolysis of ATP and the release of its products. This produces a force which causes sliding of the thin filaments in between the thick filaments, if the load is not too high, and hence muscle shortening. This well known swinging crossbridge model of muscle contraction was first put forward in 1969 by Hugh Huxley (Huxley 1969) and since then a large body of work has built up in support of this theory. However, there are certain aspects of the theory which require more clarification, for example: what are the occupancies of the different states of the crossbridges during this cycle and what are the kinetics of the movement between them?

To this end, this project is applying time-resolved X-ray diffraction to intact, active, bony fish muscle. X-ray diffraction using synchrotron radiation is particularly suited to this sort of application because it can be used on live contracting muscle specimens, with high time resolution whilst still providing data with iter-order resolution. The choice of bony fish muscle for this work arises from the fact that it is very well ordered compared to other vertebrate muscles such as frog or rabbit, showing long range order and a simple 3-D crossbridge lattice (Luther, Munro et al. 1981). For this reason, bony fish muscle produces well sampled X-ray diffraction data that can be more rigorously analysed than that from other vertebrate muscles (Harford and Squire 1986). As the muscle contracts the intensities of the reflections in the X-ray diffraction pattern change as protein mass moves within the muscle sarcomere. Analysis of these changes should lead to an understanding of the moelcular movements involved in contraction.

The sarcomere is the repeating unit of striated muscle. Many sarcomeres arranged in series form a myofibril which can extend through the whole length of the muscle cell. Many myofibrils side by side form a muscle fibre. The sarcomere contains the full molecular apparatus to perform contraction. It consists of a set of myosin filaments anchored at their centre by the M-band interdigitating with two sets of actin filaments. These in turn are anchored to Z-lines at each end of the sarcomere (see figure 1). Sarcomere length is measured from one Z-line to the next. During contraction the sarcomere shortens as the two sets of filaments slide in between each other.

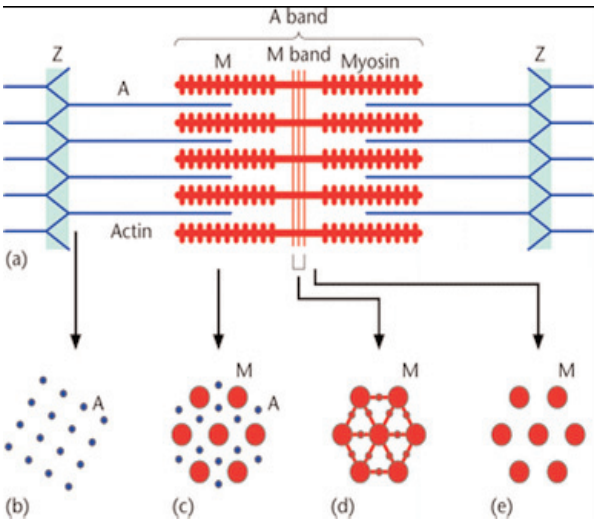

***Figure 1**: Various regions of the muscle sarcomere shown in axial view (a) and in transverse section: (b) at the Z-line (Z-band), (c) in the overlap region of the A-band, (d) at the M-band and (e) in the non-overlap region of the A-band. (A, M = actin, myosin filaments).*

Changes in the Muscle X-ray Diffraction Pattern Accompanying Contraction

i The reflections on the meridian of the pattern, labelled M3, M6 and M9 in Figure 2 are from myosin filaments. The reflections get broader and their relative intensities change.

ii The intensities of the equatorial reflections change. These give information about the muscle in a view down the filament (fibre) axis. In particular: the (1,0) reflection gets weaker and the (1,1) reflection

gets stronger as the distribution of mass within the unit cell changes.

iii The myosin layer lines, which are lines of intensity parallel to the equator due to myosin (the brown reflections in Figure 2), get weaker as crossbridges move away from theor ordered arrangement on the myosin filaments to bind to actin in active muscle.

iv The actin layer lines (the blue reflections in Figure 2) get stronger due to binding of myosin heads onto the actin filaments, although not as much as in rigor muscle where all the myosin heads bind to actin.

This reciprocal change in strength between the two sets of layer lines occurs because in relaxed muscle the myosin layer lines come from the ordered array of myosin heads on the filament backbone, but as they move to attach to actin they loose the helical repeat of the myosin filaments and reinforce the helical repeat of the actin filament.

The intensity changes which occur during contraction, in particular those on the equator of the pattern, can be used to model the crossbridge cycle. They provide information about the changing occupancies of different states in the cycle and the kinetics of the transitions between them. Previous work carried out on bony fish muscle has looked at these changes in conjunction with the tension timecourse produced by the muscle (Harford and Squire 1990; Harford, Chew et al. 1991; Harford and Squire 1992). However, factors other than movements of protein domains can affect the intensities of the reflections during contraction. In particular sarcomere length changes can also affect the timecourse of tension development. Up until now the effects of any sarcomere length change which might occur during contraction of this muscle type have been neglected, because the change was thought to be small (Harford and Squire 1992). However, it is important to be able to measure and control the sarcomere length change which occurs during contraction of bony fish muscle to quantify these effects.

With this in mind, a sarcomere length control system has been developed based on monitoring muscle sarcomere length using laser diffraction. The system can measure the sarcomere length change accompanying contraction, approximately 4% reduction in length per sarcomere, that is 47.5nm/half sarcomere, and with a feedback system can also reduce this change by about half, whilst X-ray data are being gathered. This control has had an effect on the tension timecourse. X-ray diffraction data from muscles under partial sarcomere length control and in improved physiological conditions, have been obtained on beamline 16.1 at the CLRC Daresbury Synchrotron Radiation Source. These data have been analysed using the new integrated CCP13 program FibreFix. New analysis tools have been added to the program, making the processing of time-resolved data much easier and more efficient (Rajkumar, Al-Khayat et al. 2005). Here we demonstrate some of the new functionality of FibreFix applied to the latest X-ray data from bony fish muscle under partial sarcomere length control.

Techniques

Data Collection

Time resolved X-ray diffraction data from 6 muscles (248 tetanic contractions) were obtained using the protocol in Figure 3, including some 1 ms time-frames, with the sarcomere length control system implemented on beamline 16.1 at the CLRC Daresbury Synchrotron Radiation Source with data collected on the RAPID X-ray detector.

X-ray Data Analysis

Several steps were involved in the analysis of time-resolved data using the new CCP13 program FibreFix to obtain time courses for reflections:

1. Blank cell subtraction and timecourse normalisation: BAK tool.

Muscles were mounted in thin plastic X-ray cells containing: 10mls of saline solution to keep the muscle alive and functioning; electrodes to stimulate the muscle to contract and a tension transducer lever arm to measure the tension produced by the muscle during contraction. The whole X-ray cell was then placed on the X-ray beam line. The cell has two very thin mylar windows on either side of the muscle, positioned to let the X-ray beam pass through with the least attenuation (Figure 4).

To remove any artefacts introduced into the X-ray diffraction patterns by the X-ray cell itself, diffraction patterns from the empty cell, without the muscle but containing the saline solution, were taken to be subtracted from the muscle X-ray patterns during analysis.

As said above, the main goal of analysing time-resolved data is to plot and analyse changes in the reflection intensities during a tetanic contraction. The count recorded by the RAPID X-ray detector for each pixel is proportional to the intensity of the X-rays incident on that pixel. However, the X-ray flux from the synchrotron is not constant, reducing with time since the last beam dump and so this needs to be compensated for. This was done with an ionisation chamber, mounted in front of the muscle, giving a total ionisation count for each frame of the timecourse, saved by the beamline acquisition system in a calibration file. The calibration file was then used to normalise the data and eliminate the effects of varying incident X-ray flux. This was done in conjunction with the removal of the blank cell using the BAK tool in FibreFix:

The equation which this tool applies to the timecourse is:

$$\text{Normalised Timecourse Blank Cell Removed} = \left(\text{Original Timecourse Ionisation Chamber Data} - \text{CBF} \times \text{Blank Cell} \right) \times \text{Average Ionisation Count for 1 Frame}$$

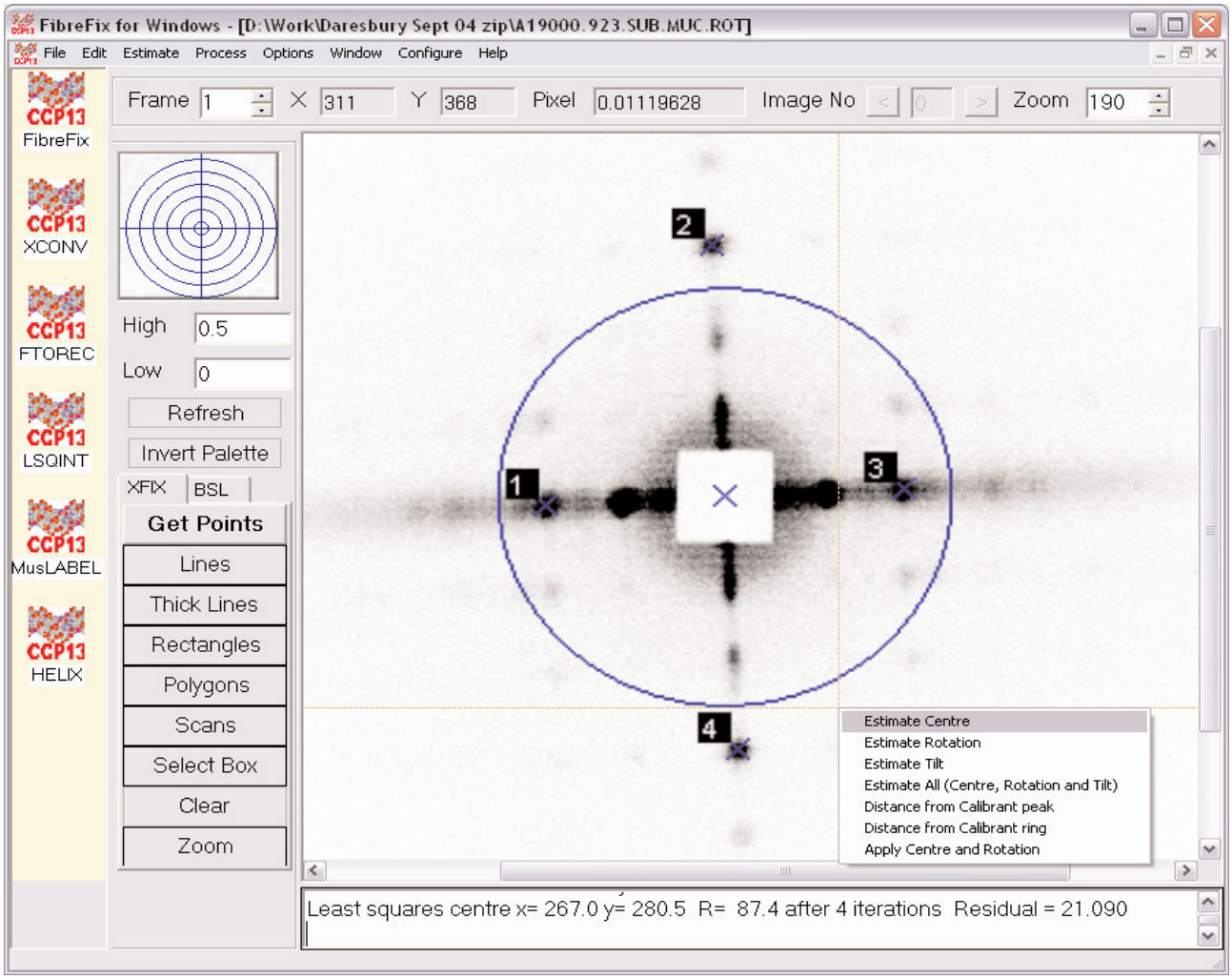

***Figure 6**: Parameter Estimation in FibreFix. See text for details.*

Where CBF is the constant background factor.

2. Finding pattern parameters and addition of time-courses

One of the disadvantages of time resolved data is that the counts for each pixel in the shortest frames are low. To improve this, timecourses from successive contractions of a particular muscle were added together and then these summed timecourses from different muscles were also added. However, each muscle was oriented slightly differently in the X-ray cell and so the centre and rotation of each summed timecourse needed to be aligned before addition.

Estimates of the centre and rotation for the summed timecourse from each muscle were obtained in FibreFix by selecting symmetric reflections around the centre of the pattern and then using the 'Estimate Centre' and 'Estimate Rotation' functions (Figure 6). These estimates were then refined using the 'Refine' function in the 'Process' menu to give good values for the centre and rotation of each summed timecourse. These parameters were applied to all frames in the summed timecourses using the 'Apply' button in the 'Parameter Editor' to centre and align them before summed timecourses from all the muscles were added frame by frame using the BSL SUM tool.

3. Rotation within the timecourse: PLA and PAK tools

Not only may the diffraction patterns from different muscle be rotated with respect to each other, but also the orientation of different frames in the timecourse from a particular muscle may occasionally change as the muscle contracts. This needs to be checked for and identified because all the frames of the timecourse must be aligned, centred and straightened before they can be added to a timecourse from another muscle. Two new tools in FibreFix were used to check for this effect: the PLA and PAK functions. The PAK tool allows all the patterns in a timecourse (or in any array of images) to be viewed at once by averaging pixels together to produce smaller images and then displaying all the frames in one image as in Figure 7. Any changes in rotation which occurred during the timecourse can be seen in this composite image. The PLA tool is of particular interest: it allows the user to scan through the time-series at speed, effectively watch-

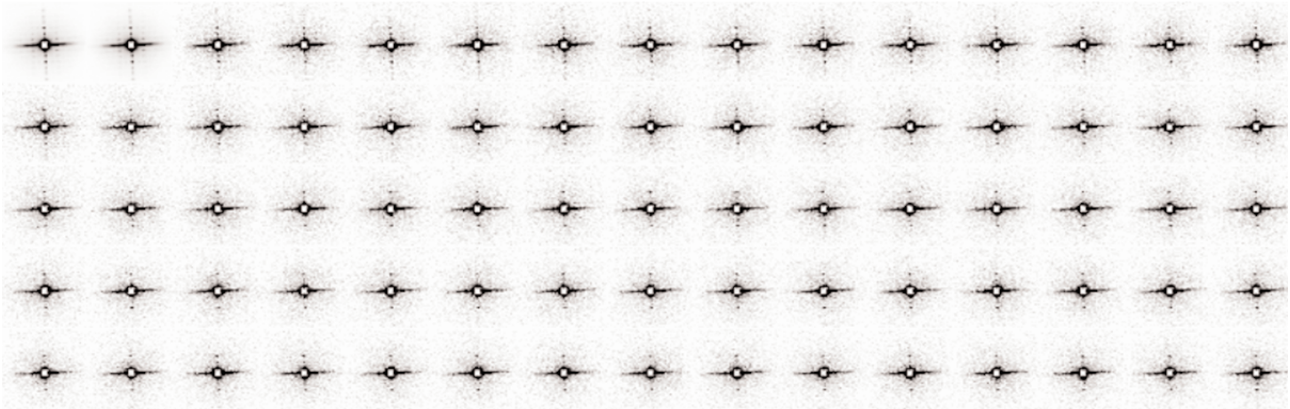

Figure 7: *Part of an image created using the PAK tool in FibreFix. Each small frame is a whole low-angle X-ray diffraction pattern from bony fish muscle (cf. Figure 8). The array can be used to assess changes in fibre orientation during a contraction.*

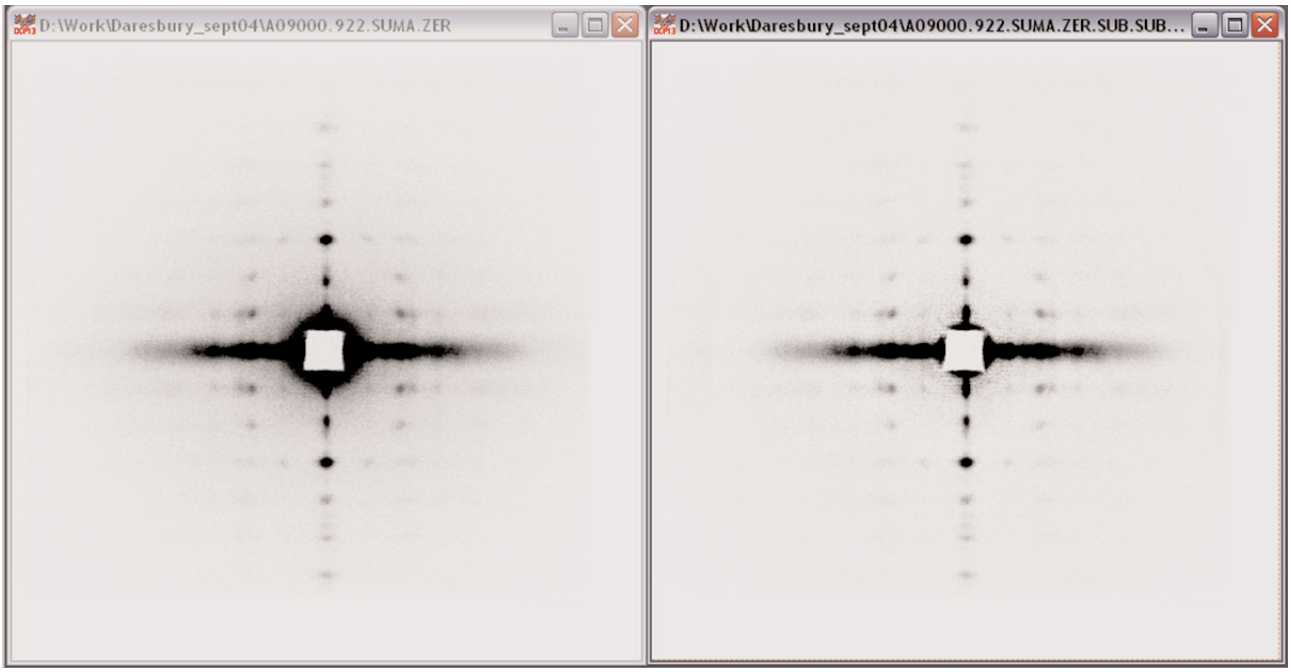

Figure 8*: Low-angle X-ray diffraction pattern from relaxed bony fish muscle before (left) and after (right) background removal. The strong meridional spot about one-third way out from the pattern centre is the M3 peak at 1/145 Å^{-1} (cf. Figure 2).*

ing a movie of the X-ray timecourse during contraction, again allowing any changes in rotation to be picked up. These differences in rotation between frames can then corrected using the BSL ROT tool which has been modified so that different rotations can be applied to different frames in the timecourse.

4. Background removal

For the correct intensities of the pattern reflections to be obtained, the background scatter of X-rays has to be removed from the total summed timecourse. FibreFix uses three different methods of background fitting: Roving Window, Circularly Symmetric and Smooth. When completed, these subtract the fitted background from each frame of the timecourse. More background fitting methods are also available in the subprogram LSQINT in FibreFix, for background subtraction later in the processing.

In the present analysis the circularly symmetric and smooth background fitting tools were used. Due to the presence of some very low counts and some pixels with zero value, the short frames (in this case the 1ms timeframes frames) in the summed timecourse were difficult to fit a background to directly. It was found that the long active frame produced the best background fit i.e. with the least amount of reflection data left in it, because it has fewer reflections in the pattern due to the myosin layer lines disappearing. Therefore, the background was fitted to the long active frame and then subtracted from all the frames in the timecourse after appropriate scaling, assuming that the background does not change greatly during

the time-course.

The smooth background fitting method was applied to the timecourse first and it was then applied again to the fitted background to remove any remaining reflection data left in the background fit. This was repeated a few times to improve the fit and the final nbackground was then subtracted from each frame in the timecourse. The circularly symmetric background tool was also used to remove the central scatter halo around the back stop.

5. Plotting reflection intensity timecourse

A new tool was developed to make this process easier in FibreFix: the TIP tool. To produce an intensity timecourse for a particular reflection in the pattern, a box was simply drawn around the reflection of interest using the TIP tool, and by clicking the right mouse button a plot was produced of the total intensity within the box with respect to the frame number. Plots for the equatorial (1,0) and (1,1) reflections were produced in this way, see figure 9.

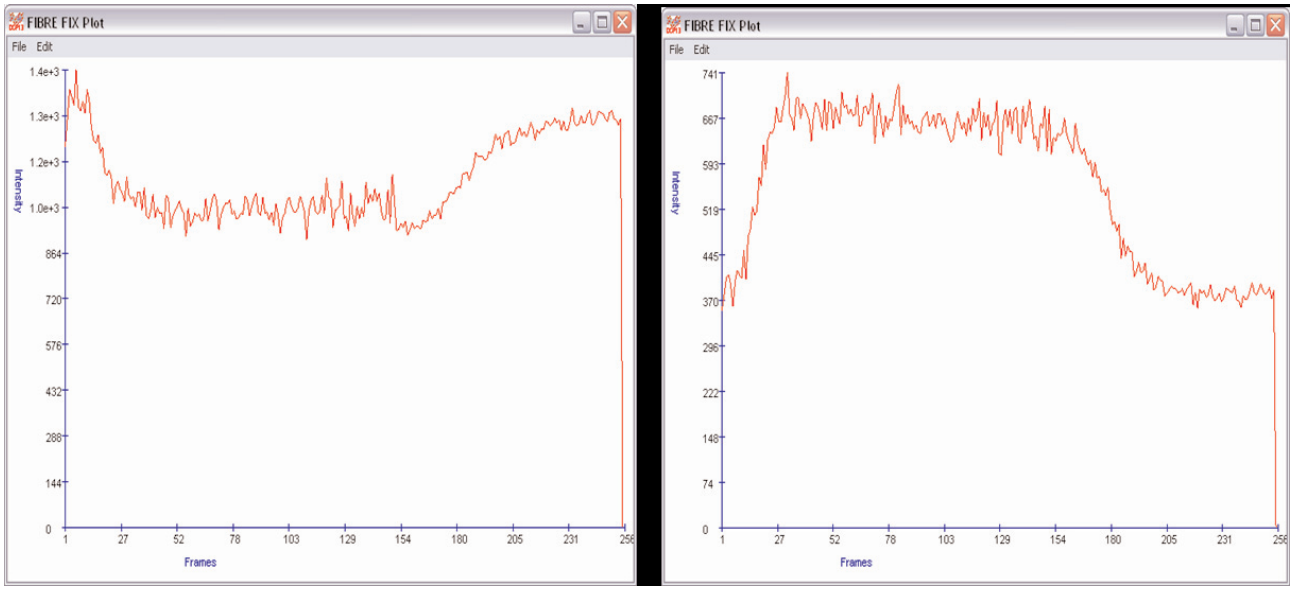

***Figure 9**: FibreFix plot of intensity changes in (1,0) (left) and (1,1) (right) equatorial reflections during the summed tetanic contraction.*

Results

Plots of the (1,0) and (1,1) equatorial reflection intensities against frame number were produced using directly from FibreFix (Figure 9). These were obtained from the X-ray timecourse of the summed tetanic contraction of 6 muscles under 50% sarcomere length control (248 tetanic contractions).

Timecourse Comparisons

The timecourses of the intensities of the (1,0) and (1,1) equatorial reflections during the first 150ms of contraction, were normalised using their average intensity during the long active frame of the timecourse. The inverse of the (1,0) reduction in intensity was taken to allow timecourses to be more easily compared. These normalised timecourses were then plotted with the normalised, average timecourse of tension development for all the summed contractions (Figure 10).

Comparing these results with previous work on vertebrate muscle, several features are of course the same (Figure 11). As tension develops:

i the (1,0) reflection gets weaker
ii the (1,1) reflection gets stronger
iii the (1,1) reflection leads tension and initily over shoots its steady state value

However, there are some features which are different. The (1,0) reflection initially follows the rise in tension closely, as in the previous data. However, it is slower to reach its active frame value than the tension timecourse, which it very closesly followed in the published timecourses. The shape of the (1,1) timecourse in the new data shows a more abrupt levelling off as the tension plateau is reached. Currently it is not clear whether these differences are due to the partial sarcomere length control present in the new data or are artefacts of the noise in the signal: more experiments are required to clarify this.

Conclusions and Future Work

A sarcomere length control system has been developed and implemented and has measured the sarcomere length change which accompanies tetanic contraction in bony fish muscle as a 4% reduction per sarcomere. The system with feedback included is also able to halve this sarcomere length change allowing X-ray data to be obtained under this control. The preliminary results presented here generally agree with the X-ray data published in previous work on bony fish. No huge changes were expected with

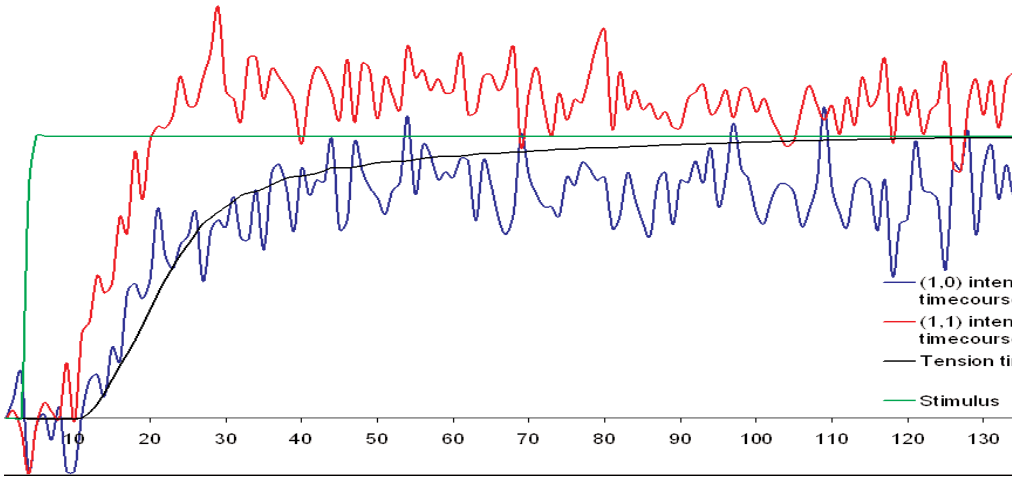

***Figure 10**: Plot of the normalised intensity changes of the blue (1,0) (inverse change) and red (1,1) equatorial reflections and the tension produced (black) during the rising phase of contraction in bony fish muscle.*

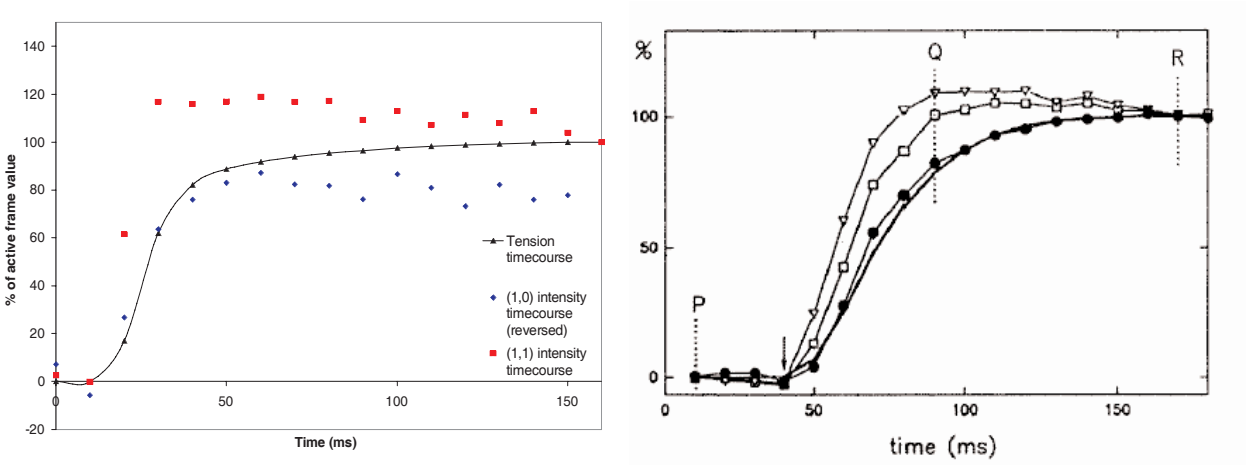

Figure 11: Comparison of normalised timecourses on a longer timebase (left) with previously published data from fish muscle (Harford and Squire 1992) (right)

the use of the partial sarcomere length control. Cecchi et al reported no obvious difference in the intensity timecourses from sarcomere length clamped and unclamped single frog fibres in their 1991 paper (Cecchi, Griffiths et al. 1991). More data are needed to clarify the differences seen in the timecourses from both reflections in this recent data from bony fish msucle.

New X-ray data have now been obtained from muscles both under partial sarcomere length control and without any control and these still need to be analysed as described here. The (1,0) and (1,1) timecourses which will be obtained from the new data, along with those from other equatorial reflections, can then be used to model the crossbridge cycle with both partially controlled and uncontrolled sarcomere length changes. This will help to determine any differences the reduction in sarcomere length change may be causing in the X-ray pattern and crossbridge cycle.

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